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Preliminary Evaluation of Antioxidant and Cytotoxic Activity of Ethanolic Extract of Stingless Bees Propolis From Different Localities

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ABSTRACT

Propolis is a resinous mixture collected from stingless bees and is reported to have various biological activities such as antioxidant, antibacterial, antitumor and hepatoprotective. Location where bees were bread plays an important role in determining the types and quality of propolis. This study aimed to investigate the effect of locality on antioxidant and cytotoxic activity of propolis. Stingless bee *Heterotrigona itama* propolis was collected from Besut (HI-BST), Dungun (HI-DGN), Tanah Merah (HI-TM) and Gua Musang (HI-GM). The antioxidant activity of these propolis was determined using 2, 2-diphenyl-1-picrylhydrazy (DPPH) and 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS). Cytotoxic and apoptotic activities of these propolis extracts were determined through 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium Bromide (MTT Assay) and Annexin V/PI double staining method using flow cytometer. HI-BST propolis sample has the highest antioxidant capacity with IC_{50} value of 10 ± 2.623 μgmL^{-1} (DPPH free radical scavenging) and 30 ± 1.857 μgmL^{-1} (ABTS free radical scavenging) followed by HI-DGN and HI-GM. For cytotoxic and apoptotic evaluation, HI-BST has the highest cytotoxic, with IC_{50} value of 14 ± 0.910 μgmL^{-1} compare to other propolis samples. In apoptosis assay, at concentration of 14 μgmL^{-1} (IC_{50}), there were 44.84% of cell undergoes early apoptosis while 38.81 % was viable cell. Overall, data from this study shown that propolis collected form university's apiary (HI-BST) is the best quality propolis compare to other location. Propolis from this location has good potential to be developed as health-related product.

Keywords: propolis, stingless bees, *Heterotrigona itama,* antioxidant, cytotoxic, apoptosis

ABSTRAK

Propolis adalah campuran resin yang dikumpulkan dari lebah (kelulut) dan dilaporkan mempunyai pelbagai aktiviti biologi seperti antioksidan, antibakteria, antitumor dan hepatoprotektif. Lokasi dimana lebah diternak memainkan peranan penting dalam menentukan kualiti propolis. Kajian ini bertujuan meneliti kesan lokasi terhadap aktiviti antioksidan dan sitotoksik propolis. Propolis dari kelulut *Heterotrigona itama* dikutip dari Besut (HI-BST), Dungun (HI-DGN), Tanah Merah (HI-TM) dan Gua Musang (HI-GM). Ekstrak etanol disediakan. Aktiviti antioksida propolis ini ditentukan menggunakan kaedah 2, 2-diphenyl-1-picrylhydrazy (DPPH) dan 2, 2-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS). Aktiviti sitotoksik dan apoptosis ditentukan melalui kaedah 3- (4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetra-zolium Bromide (MTT esei) dan juga ujian dwi-pewarnaan Annexin V/PI melalui kaedah flow-sitometri. HI-BST mempunyai kapasiti antioksida tertinggi dengan nilai IC_{50} 10 ± 2.623 μgmL^{-1} (pemerangkap radikal bebas DPPH) dan 30 ± 1.857 μgmL^{-1} (pemerangkap radikal bebas ABTS) diikuti oleh HI-DGN dan HI-GM. Bagi penilaian sitotoksik dan apoptosis, HI-BST mempunyai nilai sitotoksik tertinggi dengan IC_{50} 14 ± 0.910 μgmL^{-1}. Manakala, untuk ujian Annexin V-FITC pada kepekatan IC_{50} (14 $\mu g\ mL^{-1}$) terdapat 44.84% sel yang mengalami fasa awal apoptosis manakala 38.81% adalah sel yang masih hidup. Secara keseluruhannya, data diperoleh dari kajian ini menunjukkan bahawa propolis yang dikutip dati apiary universiti (HI-BST) adalah propolis paling berkualiti berbanding lokasi lain (HI-DGN, HI-GM and HI-TM). Propolis dari lokasi ini mempunyai potensi yang baik untuk dibangunkan sebagai produk berkaitan kesihatan.

Kata kunci: propolis, kelulut, *Heterotrigona itama,* antioksida, sitotoksik, apoptosis

INTRODUCTION

Propolis is a mixture of plant exudates collected by bees to fix the hole in the beehive (Marcucci et al., 2001). Stingless bee uses propolis to build the entire hive including their holey pots. Physically, propolis is a sticky gum with variable color from yellow mix with green to dark brown and it is depend on its age and source. Propolis sometimes referred as "bee glue". Study on honeybee propolis around the world revealed that propolis composed of at least 300 compounds. The main compositions are resin (50 %), wax (30 %), essential oils (10 %), pollen (5 %), and other organic compounds (5 %) (Castro, 2011; Gomez-´Caravaca et al., 2006). Propolis also

found having many benefits such as antibiotic, antifungal, antiviral and antitumour properties (Fatoni et al 2008; Reynaud et al 2005; Trusheva et al 2006; Jin et al 2005). In Malaysia, there are two common stingless bee (locally known as kelulut) species, *Heterotrigona itama* and *Geniotrigona thoracica,* which are the main pollinator in this region. In comparison to honeybee, stingless bee cannot produces large amount of honey, but they are able to produce substantial quantity of propolis compare to other bee species (Ibrahim et al., 2016). It is believed that stingless bee propolis possess more therapeutic property compare to that of honeybees. The complexity of propolis chemistry is one of the challenges in pinning down the efficacy of propolis. Location, types of vegetation, species are all contribute to the quality of propolis. Studies have shown that propolis activity from different geographical origin possessed different chemical compositions (Markham et al., 1996). Our previous study (Ibrahim et al 2016) showed that propolis collected by *Heterotrigona itama* has better antioxidant and antibacterial activity compare to *Geniotrigona thoracica*. This main focus of this study was to investigate biological activities of the propolis produced by *Heterotrigona itama* that were collected from various localities of Dungun (Terengganu), Tanah Merah (Kelantan), Besut (Terengganu) and Gua Musang (Kelantan).

MATERIALS AND METHODS

Sample preparation

Propolis was collected from four locations in east coast region of Peninsular Malaysia, which were Besut (Terengganu), Dungun (Terengganu), Tanah Merah (Kelantan) and Gua Musang (Kelantan). Types of vegetation surrounding the area are shown in Table 1. The samples were frozen at -80°C, ground using wearing blender and kept in -80°C for further analysis. Approximately, 18 g of propolis was added into 60 mL of 95 % ethanol and macerated at room temperature for 3 days. After that, the solutions was filtered and concentrated by using rotary evaporator (Heidolph, Germany) under vacuum pressure at temperature of 45°C. The extracts were kept at 4°C prior analysis. The samples of crude propolis were labeled as HI-BST, HI-TM, HI-DGN and HI-GM, as shown in Table 1.

Table 1 The location of the HI-BST, HI-DGN, HI-TM and HI-GM propolis samples

Geographic Origin	Sample Code	Plant Sources
Besut *(N 5° 45' 34.6" N 102° 38' 18.5"E)*	HI-BST	*Acacia sp.* (Akasia), *Melaleuca cajuputi* (Gelam putih), *Melaleuca viridiflora* (Gelam Merah), *Synsepalum dulcificum* (Pokok ajaib) & *Antigonon leptopus* (Air mata pengantin).
Dungun *(N 04° 42' 43.7" N 103° 23'49.3"E)*	HI-DGN	*Acacia sp.* (Akasia), *Melaleuca cajuputi* (Gelam putih), fruits, *Bamboo sp.* (Buluh) & shrub.
Gua Musang *(N 04° 54' 40.9" E 102° 10' 23.8")*	HI-GM	*Lansium domesticum* (Duku) & Durian.
Tanah Merah *(N 05° 53' 23.2" E 102° 09' 27.4")*	HI-TM	*Salacca zalacca* (Buah salak), *Hevea brasiliensis* (Pokok getah), *Cocos nucifera* (Buah kelapa) & *Cocos nucifera* (Buah kelapa).

Antioxidant activity

DPPH assay

In this assay, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay used to measure radical scavengers of the extracts in accordance to Ibrahim et al (2015) with some modification. About 5 mg/mL of the stock solution of standard (trolox) and propolis extracts were dissolved in 1 mL of dimethyl sulfoxide (DMSO). Standard and samples were diluted in 96-well-plate from the stock solution to produce final concentration of 7.81, 15.63, 31.25, 62.5, 125, 250, 500 μg/mL. Propolis and standards were then mixed with 0.125 mM DPPH in methanol, to produce a final DPPH concentration of 0.1 mM in final volume of 250 μL in each well. Absorbance was taken

at 517 nm after 30 min incubation at room temperature in the dark. The percentage of inhibition was calculated using the following formula:

Percentage of Inhibition (%): 1-(A517nm, samples/A517nm, control)*100

ABTS assay

ABTS radical cation ($ABTS^+$) scavenging activity was determined according to the method described by Shalaby & Shanab (2013) with some modification. This method measures antioxidant activity of both water-soluble and lipid soluble antioxidants, as well as extracts from natural sources (Erkan et al., 2008). $ABTS^+$ produced by reacting 38.43 mg ABTS (final concentration of 7 mM) and 6.90 mg potassium persulfate (final concentration of 2.55 mM) and 10 mL demineralised H_2O in an Erlenmeyer flask and keeping the mixture in the dark at 26 ± 3ºC for 12 hours. An aliquot of blue–green $ABTS^+$ solution was obtained by diluting the prepared mixture with 95 % ethanol to give an absorbance of 0.70 ± 0.02 at 734 nm. $ABTS^+$ adjusted solution (1 mL) and ethanol (0.02 mL) were mixed in a semi-microcuvette and absorbance at 734 nm corresponding to blank (E1) was recorded (Spectronic Genesys 5, Germany). Test sample (0.02 mL) was then added into semi-microcuvette. The absorbance of each extract was recorded. The reaction mixture was allowed to stand for exactly 6 min (E2). The percentage of $ABTS^+$ scavenged was calculated as:

ABTS scavenge % = ((E1-E2)/E1)*100

Cytotoxic activity

Cell culture condition

Cell culture protocol was carried out in accordance to Yaakob (2017) with slight modification. Human Cervical Carcinoma (HeLa) cell lines were supplied with DMEM media, supplemented with 10 % of Fetal Bovine Serum (FBS) and 1 % of antibiotic (pen/strep). Cells were incubated at 37°C supplied with 5% CO_2 and 90% humidity. Media was replaced every two days.

MTT assay

Cytotoxicity of propolis extract against HeLa cells was determined by using 3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT) assay adapted from Choudhari et al. (2013) with some modification. A volume of 100 µL of cells with media were plated in 96-well tissue culture plate and were incubated for 24 hours at 37°C in atmosphere of 5% CO_2 and 90% humidity in the CO_2 incubator. Thereafter, the cells were treated with different concentrations (10, 25, 50, 100, and 250 µg/mL) of propolis extract for 24 hours. Doxorubicin served as positive control. Cells cultured in the absence of extract served as negative control. Cytotoxicity were assessed by adding 20 µL of MTT (5 µL of MTT/well) solution into 96-well plate, followed by incubation at 37°C for 4 hours. Absorbance was measure at 570 nm. The percentage of cell viability was calculated by using the following formula:

Percentage (%) of cell viability = (OD570 treated cells/ OD570 control)*100

Determination of apoptotic cell death by annexin V-FITC

Apoptotic assay was carried out by method adapted from Paredes-Gamero (2012). Approximately 1.5 x 10^5 cells/mL of HeLa cells were incubated for 72 hours with propolis extracts. Cells were then harvested and spun for about 10 minutes at 300x g. Cells were then washed twice with PBS and were added with 100 µL of 1X binding buffer. The cell suspension was then incubated with 1.25 µL of annexin V-FITC and 1.25 µL of propidium iodide (PI) solution for 15 minutes at room temperature in the dark. A volume of 400 mL of 1X binding buffer was added to the cell suspension and mixed. The annexin V assay was performed using the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickenson). About 10,000 cells were sorted using flow

cytometer (CytoFLEX, Beckman Fourier) and analyzed using CytoExpert software. Doxorubicin used as positive control.

Data Analysis

The data were expressed as means ± S.D, which obtained from four separate experiments. Statistical significance was evaluated using two way ANOVA, at which *P*-values less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Antioxidant activity

DPPH radical scavenging assay is a common method to evaluate radical scavenging activity of antioxidant. The DPPH was developed by Blois which basically aiming to determine the antioxidant activity by using a stable free radical, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M=394.33). In this assay, the inhibition capacity of particular antioxidant molecule towards the DPPH was measured. By which, DPPH donor its electron of nitrogen atom and received a hydrogen atom from antioxidants (Contreras-Guzman and Srong 1982). The solution then turned into a deep violet colour and decolorization is stoichiometric with respect to the number of electrons taken up (Kedare and Singh, 2011). This method is suitable to work in both aqueous and nonpolar organic solvents. It also be able to analyze with both hydrophilic and lipophilic antioxidant molecules (Prior et al. 2005). From Figure 1, it shows that most of the propolis samples are able to inhibit DPPH free radical more than 70 percent except for HI-TM sample.

Table 2 shows that quercetin has IC_{50} values of 4 ± 2.623 µg/mL). The HI-BST possesses the lowest IC_{50} values (10 ± 2.623 µg/mL) followed by HI-DGN (84 ± 2.623 µg/mL) and HI-GM (151 ± 2.623 µg/mL). While, HI-TM propolis sample shows the highest IC_{50} values (280 ± 2.623 ug/mL). The lower the IC_{50} value of scavenging activity indicates the stronger the antioxidant activity. Data from this experiment revealed that propolis collected from Besut have strongest antioxidant activity and propolis from Tanah Merah have the least antioxidant activity of DPPH scavenging radicals. This result corroborated with report by Ibrahim et al. (2015) where they show *H. itama* propolis from Besut Apiary has highest antioxidant activity.

The ABTS assay is based on the formation of the ferrymyoglobin radical from metamyoglobin and hydrogen peroxide. This compound then oxidizes phenothiazine compound 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) to produce a radical cation ($ABTS^+$) and producing the blue-green solution. $ABTS^{\bullet+}$ has strong absorption at longer wavelengths (with absorption maxima at 415, 645, 734, and 815 nm), but 734 nm is adopted by most investigators. In the absence of antioxidant, ABTS• is rather stable, but reacts energetically with molecules able to donate hydrogen atoms or electrons, such as phenolic compounds, leading to a disappearance of the blue/green color of this radical. The decolorization is stoichiometric with respect to the number of electrons taken up (Kedare and Singh, 2011).

Table 2 Fifty percent of inhibition concentration of propolis extracts and standard by DPPH free radical scavenging assay.

Sample	IC_{50} (µg/mL)
HI-TM	280
HI-DGN	84
HI-GM	151
HI-BST	10
Quercetin	4

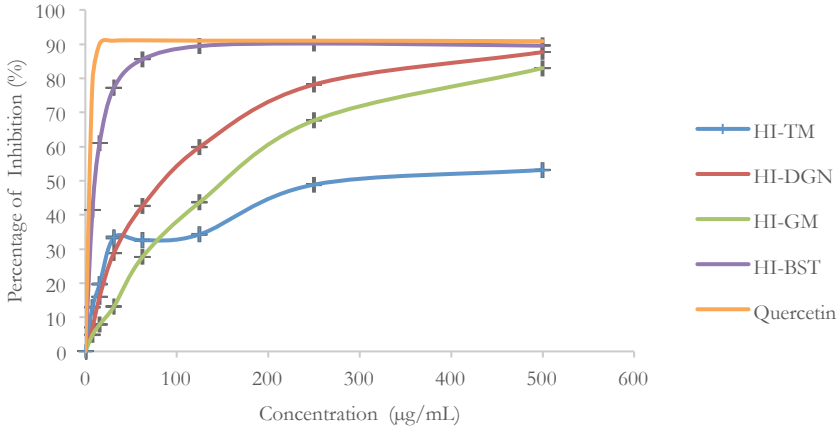

Figure 1 DPPH free radical scavenging activity of propolis extract. HI-BST-Sample from Besut; HI- DGN-Dungun; HI-TM- Tanah Merah; HI-GM- Gua Musang. Quercetin served as antioxidant standard.

ABTS•+ can be solubilized in both aqueous and organic solvents, and thus multiple media can be used to determine both hydrophilic and lipophilic antioxidants. From Figure 2, it shows that most of the samples able to inhibit ABTS free radical more than 70 percent. As shown in Table 3, the standard assessed, ascorbic acid has IC_{50} values at 5 ± 1.857 µg/mL. Among all propolis samples, HI-BST shows the lowest IC_{50} values (30 ±1.857 µg/mL) followed by HI-DGN (52 ± 1.857 µg/mL and HI-GM (75 ± 1.857 µg/mL). While, HI-TM shown the highest IC_{50} values (80 ± 1.857 µg/mL). In agreement with DPPH radical scavenging capacity, HI-BST also has the strongest scavenging activity of ABTS radical scavenging capacity. The activeness of an antioxidant activity of a particular compound depends on its molecular structures (Rice-Evan et al., 1996). Compounds that have more than one active group (e.g., NH2 or OH) in ortho position are good antioxidant agent. For example, the phenolic compound is widely known to break the antioxidant chain with the help of hydroxyl groups (Sawant et al., 2009).

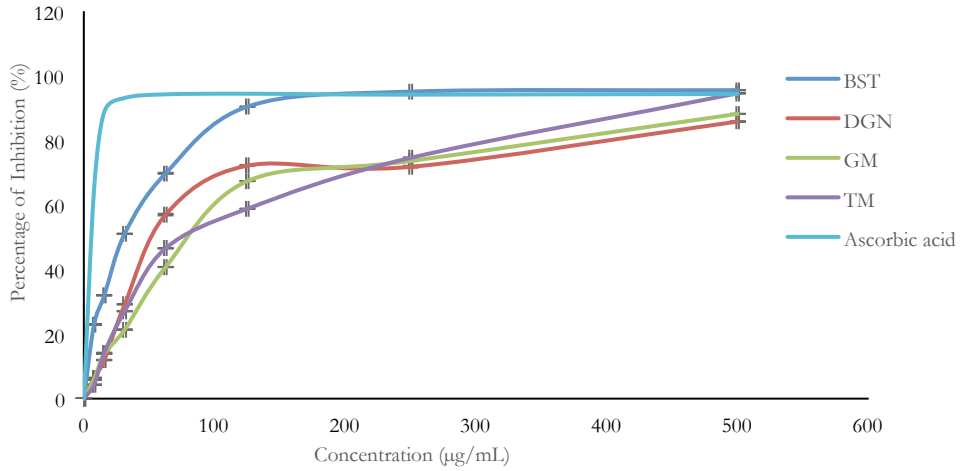

Figure 2 ABTS free radical scavenging activity of propolis extract. HI-BST-Sample from Besut; HI- DGN-sample from Dungun; HI-TM- sample from Tanah Merah; HI-GM- sample from Gua Musang. Quercetin is antioxidant standard.

Table 3 Fifty percent of inhibition concentration of propolis extracts and standard by ABTS free radical scavenging assay.

Sample	IC_{50} (µg/mL)
HI-TM	80
HI-DGN	52
HI-GM	75
HI-BST	30
Ascobic acid	5

Cytotoxic and apoptotic evaluation of various propolis samples

In order to investigate cytotoxic effects of different propolis from different location, propolis extracts were screened against HeLa cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assay. The IC_{50} values of all samples are shown in Figure 4 and Table 4. Doxorubicin has served as positive control, where IC_{50} value determined to be 1 ± 0.910 µg/mL. HI-BST propolis has moderate cytotoxicity against HeLa cells with IC_{50} values of 14 ± 0.910 µg/mL. HI-DGN and HI-GM showed to have weak cytotoxicity activity against HeLa with IC_{50} of 32 ± 0.910 µg/mL and 38 ± 0.910 µg/mL, respectively. HI-TM shows weak toxicity against HeLa cells with IC_{50} values of 60 ± 0.910 µg/mL

Cytotoxic screening revealed that HI-BST has a moderate cytotoxic effect against HeLa cells. In order to determine the mode of cell death caused by HI-BST extract, flow cytometry analysis on double staining Annexin V-FTIC was performed.

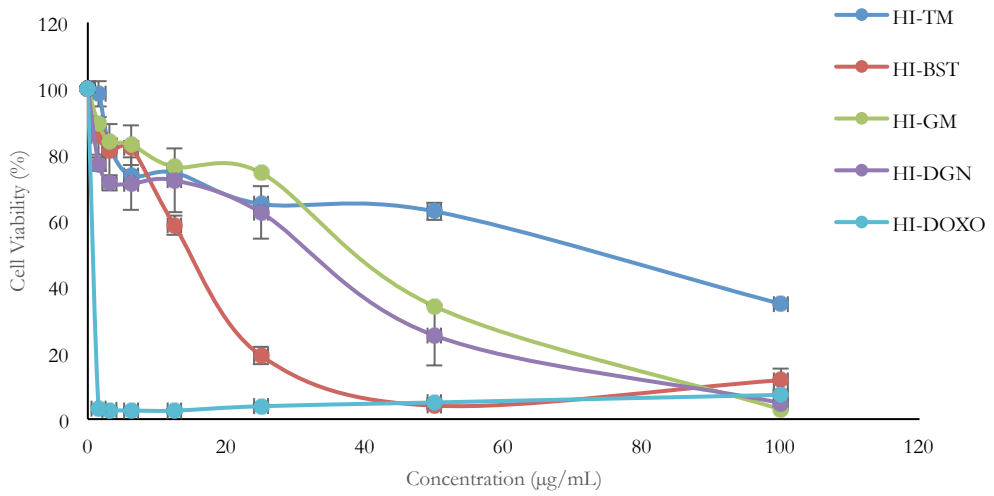

Figure 3 Cytotoxic activity of propolis extracts against HeLa cell lines. HI-BST-Sample from Besut; HI- DGN-sample from Dungun; HI-TM- sample from Tanah Merah; HI-GM- sample from Gua Musang. Doxorubicin is a positive control.

In Annexin V-FITC technique, Annexin V exhibits anti-phospholipase activity and attached to phosphatydilserine. Annexin V is attached to negatively charged phosphatidylserine (PS) in the present of calcium cation. PS is located at the outer membrane, which face the cytosol. During apoptosis, the cell loss membrane integrity, which result in exposing the PS to the outer cell membrane (Koopman et al., 1994). Consequently, allows Annexin V to attach at the exterior of apoptotic cells. Flow cytometry is a modern technology that provides the information on cell proliferation and cell loss. It is a rapid and capable of analyze multiparameter of large cell populations. At early stage of apoptosis, the alteration of cell size and shape can be detected following the alterations in the forward (FSC) and side scatter (SSC) parameters (Lecoeur et al., 1997).

Table 4 Fifty percent of inhibition concentration of propolis extracts and standard of cytotoxicity activity by MTT assay against HeLa cell lines.

Samples	IC $_{50}$ (µg/µL)
HI-TM	60
HI-DGN	32
HI-GM	38
HI-BST	14
Doxorubicin	1

FSC detects alteration in cell size, whereas the modification in SSC provides the information of fragmentation, extreme condensation, or blebbing of the cell (Frey, 1997). As seen in Figure 4(A), the box was consisted of four sections. The lower left quadrant for the viable cell while for the right one, shows early apoptosis event. Both upper right and left indicate the late apoptosis and necrosis, respectively. The concentration of viable cell was decreased with the increasing of the concentration of HI-BST. Without the treatment of HI-BST propolis sample, the HeLa cells were 100 % viable.

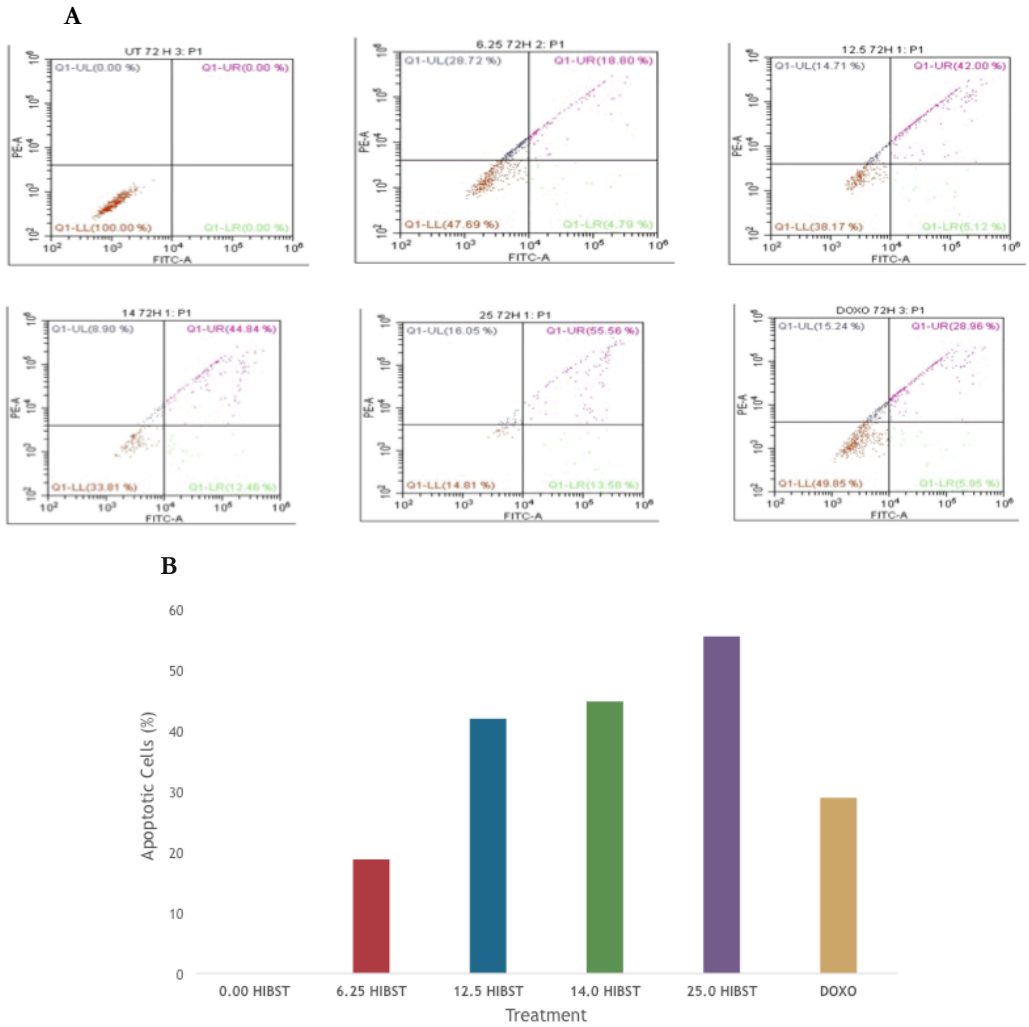

Figure 4 Flow cytometric analysis of Annexin V/PI double staining of HeLa cells treated with various concentration of HI-BST. (A)- Distribution of cells sorted by flow cytometer. (B)- The percentage of apoptotic cell at various concentration of HI-BST.

This indicates that the cells were in healthy condition. At concentration of 6.25 μg/mL, there were 47.69% of viable cells and 18.18 % of early apoptosis cells. At concentration of 12.5 μg/mL, there were 38.17% of viable cells and 42 % of early apoptosis cells. At the concentration IC_{50} (14 μg/mL) there were 44.84 % of cells in early apoptosis while 38.81% were viable cells. At the concentration of 25 μg/mL shows that about 55.56 % of early apoptosis cell with 14.81 % of viable cell. All samples have a significantly difference ($p< 0.05$), except for relation between HI-TM and HI-GM. For HI-TM and HI-GM, their percentage of inhibition was not significant (p value>0.05).

Honeybee propolis has the ability to induce apoptosis through the release of cytochrome c from mitochondria to the cytosol, activation of caspase cascade and TRIAL signal (Sawicka, 2012). Propolis was able to induce apoptosis in tumor cells due to the present of two active compounds, CAPE and chrysin. These two compounds have been tested in a variety of culture cell lines. In fact, majority cytotoxic activity of propolis is the resulted of the presence of CAPE in propolis. However, it is yet to determine on whether the same compounds occur in Malaysian stingless bee propolis. This study shows that localities play crucial role in determining the quality of propolis. Propolis from Besut (HI-BST) has the highest potential in terms of antioxidant capacity and cytotoxic activity. The differences in biological activity could be due to the differences in chemical compounds, which might be contribute from different type of vegetation surround the vicinity. It is remain unclear whether bioactive compounds such as CAPE and crysin occur is Malaysian plants

CONCLUSION

Based on the result obtained, propolis from Besut (HI-BST) has the highest scavenging activity against both ABTS and DPPH free radical. The HI-BST also shows the highest cytotoxic activity against HeLa cells compare to other locations. In addition, HI-BST was capable of inducing apoptosis in HeLa cells. The finding of this study indicates that Besut has the potential to become breading area for *Heterotrigona itama* and produce high quality propolis that can be developed as health-related products.

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